ISSN 0126-0472 EISSN 2087-4634
Accredited by DGHE No: 66b/DIKTI/Kep/2011

Media Peternakan, August 2014, 37(2):95-100
DOI: 10.5398/medpet.2014.37.2.95
Available online at http://medpet.journal.ipb.ac.id/

The Quality of Spermatozoa of Gembrong Goats during Cryopreservation Process

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(Received 16-04-2014; Reviewed 02-06-2014; Accepted 14-07-2014)

ABSTRACT

Gembrong goat is an Indonesia local goat having specific characteristic that is currently categorized as a breed that is at risk of extinction. In this context, the cryopreservation of gametes is important to support a genome resource bank for storage of gametes for an indefinite period of time. Evaluation of semen and spermatozoa quality was performed to determine the survival of spermatozoa and this information will be used as a reference in the cryopreservation of semen and spermatozoa. The aim of this experiment was to study the characteristics of Gembrong goat's semen and spermatozoa during cryopreservation process. Once a week, semen from three Gembrong goats (ages about 2-3 years old) was collected using artificial vagina and then frozen with TRIS extender. After freezing, the semen was thawed. Macro- and microscopic parameters of semen and spermatozoa were assessed in fresh and frozen-thawed semen. Results showed that in the fresh semen, the volume was 0.5 mL, sperm abnormalities was 5.74%, sperm concentration was 6731 x 10^6/mL, the sperm motility was 78.33%, live sperm was 83.17%, and sperm membrane integrity was 78.53%. After-thawing observation showed that sperm motility decreased to 49% (P<0.05) that was lower as compared to that in the fresh and post-equilibration semen. Similarly, the percentage of sperm viability and membrane integrity during cryopreservation showed a similar pattern with the sperm motility. In conclusion, the fresh semen of Gembrong goat had a good quality and met the requirement for further cryopreservation process. Similarly, the quality of frozen-thawed semen of Gembrong goat is eligible for artificial insemination (AI) or *in vitro* embryo production.

Key words: gembrong goats, sperm cryopreservation, sperm quality

ABSTRAK

Kambing Gembrong merupakan salah satu jenis kambing Indonesia yang memiliki penampilan yang spesifik dengan status terancam punah. Kriopreservasi spermatozoa diperlukan sebagai usaha pembentukan bank sperma dalam rangka menyelamatkan materi genetik hewan jantan dikarenakan pemanfaatannya dalam jangka panjang. Evaluasi kualitas spermatozoa dilakukan untuk menentukan daya hidup spermatozoa dan digunakan sebagai acuan dalam kriopreservasi spermatozoa. Tujuan penelitian adalah untuk mengetahui karakteristik spermatozoa kambing Gembrong selama proses kriopreservasi. Sebanyak 3 ekor kambing Gembrong yang berumur 2-3 tahun ditampung semennya satu kali seminggu menggunakan vagina buatan, dibekukan dengan pengencer TRIS, lalu di-*thawing*. Evaluasi karakteristik spermatozoa, baik makroskopik maupun mikroskopik, dilakukan pada semen segar, pascaekuilibrasi, dan pasca-*thawing*. Hasil penelitian menunjukkan bahwa volume semen segar adalah 0,5 mL; abnormalitas 5,74%; konsentrasi 6731x10^6/mL; motilitas 78,33%; viabilitas 83,17%; dengan integritas membran 78,53%. Setelah *thawing,* motilitas spermatozoa mengalami penurunan yang cukup drastis menjadi 49% dan secara nyata (P<0,05) lebih rendah dibandingkan pada tahap segar dan ekuilibrasi. Demikian pula pada persentase viabilitas dan integritas membran spermatozoa selama kriopreservasi menunjukkan pola yang sama dengan motilitas. Disimpulkan bahwa kambing Gembrong memiliki kualitas spermatozoa yang baik dan memenuhi syarat untuk proses kriopreservasi. Demikian pula karakteristik spermatozoa kambing Gembrong selama proses kriopreservasi dan *post thawing* masih layak digunakan untuk inseminasi buatan (IB) atau produksi embrio *in vitro*.

Kata kunci : kambing gembrong, kriopreservasi, spermatozoa, kualitas spermatozoa

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INTRODUCTION

Goat is one of the biodiversity resources in Indonesia that has a potential to be developed and conserved as a meat-producing animal. Goat is one of the animal breeds that contribute in food supply, especially in animal-protein production, for human consumption (Handiwirawan *et al*., 2007). In Indonesia, it is reported that at least there are 13 breeds of goat, both local and crossed bred goats, which are distributed in all Indonesian archipelago. Gembrong goat is one of goat breeds having specific characteristics. In Indonesia, this breed of goat is only found in Bali, mainly in Karangasem Regency. The existence of this breed contributes to biodiversity in Bali so that this breed of goat is needed to be conserved as a local asset. There is no available scientific data as a reference to the origin of this goat (Zein *et al*., 2012).

However, for the time being, population of Gembrong goat is decrease continuously so that if it is not handled seriously, it would end up in a distinction that would cause a loss of animal-genetic resources. Some factors causing the drastic decrease in population of Gembrong goat is an extensive management system that is only grazed with the very minimum maintenance without a good disease control and reproduction program (Oka *et al*., 2011). In addition, another problem that is frequently happened in the field is the predation by forest dogs that frequently predate the goat in the evening. These factors cause the critical status and even can cause an extinction of the local goat (Oka *et al*., 2011). In addition, the genetic materials of the animal could be lost any time due to the unexpected mortality of the goat, the low libido, or the problem in the reproductive tract (Drouineaud *et al*., 2003; Kaabi *et al*., 2003). The loss of genetic material of Gembrong goat will affect the local animal biodiversity because the lost is irreversible and the genetic potential of Gembrong goat is still unknown.

The effort that can be done to avoid the loss of genetic materials of the animals is by conserving and saving the genetic materials so it can be used in the future for artificial breeding by a technology-assisted application. One of effort that can be used in preserving and conserving germ plasma of male animals is by cryopreservation of the semen or spermatozoa. Cryopreservation of semen or spermatozoa is a technique to preserve and store the spermatozoa in a frozen condition (at -196 ^{o}C temperature) that involves some factors such as a correct extender, the process of semen dilution, the speed of cooling, freezing, and thawing in addition to the understanding of spermatozoa physiology and the quality of semen and spermatozoa in certain animal species (Purdy, 2006). Cryopreservation is a method that can be used to support the conservation of animal's genetic materials by freezing the semen and the use of the conserved semen and spermatozoa is not limited by geographical distance and time (Martins *et al*., 2007).

Evaluation of sperm quality is conducted to document the viability of the spermatozoa after cryopreservation and to standardize a technique for cryopreservation of semen and spermatozoa and its application for the technology-assisted animal reproduction such as artificial insemination. Therefore, this study was conducted to study the characteristics of semen and spermatozoa of Gembrong goat in fresh condition at the time of semen collection and after cryopreservation process.

MATERIALS AND METHODS

The experiment was conducted in the Reproduction Laboratory of Loka Penelitian Kambing Potong Sei Putih, North Sumatera Utara. Three pure breed male Gembrong goats, aged 2-3 yr and weight ranged 35-40 kg, obtained from the ex situ collection in Loka Penelitian Kambing Potong were used in the experiment. The experimental goats were maintained in the individual cage with feeder and drinking water. Feed was provided in the form of concentrate and grass. Concentrated was given in the morning feeding around 300-500 g per goat per day and fresh grass was given at noon and in the afternoon with the total of 3-4 kg per goat per day. Drinking water was provided *ad libitum*.

Semen Collection and Cryopreservation

The semen was collected from each male Gembrong goat once a week (1 ejaculate for each collection period) with 5 wk replications. Semen collection was conducted by using artificial vagina with inner liner and was filled with water with temperature of 40-42 ^{o}C. The artificial vagina was filled with air by valve and was coated with lubricant with the deep of not more than 3 cm so that the artificial vagina had the similar condition with the real vagina. At the other end of the artificial vagina, the tube was set up for semen collection. The collected fresh semen was then evaluated for further processing.

Cryopreservation was conducted by using extender with composition of 2.96 g Tris amino methane, 1.65 g citric acid, 2.16 g lactose, 6 mL glycerol, 1000 IU/mL penicillin, 1000 μg/mL streptomycin, 20 mL egg yolk, and 100 mL aquabidest ad (Kostaman *et al*., 2000). After dilution, the final sperm concentration of semen after dilution was 100 x 10^{6}/mL, and was then equilibrated at temperature of 5 ^{o}C for 2 h. Then, the semen was put into a straw with the size of 0.25 mL (IMV, France), and then was put in a styrofoam plate in the liquid nitrogen steam for 20 min (around 4 cm from the surface of the liquid nitrogen) and then soon was put into a liquid nitrogen container for storage. The thawing was conducted by placing the straw of frozen semen in the air at room temperature for 30 seconds and then it was dipped in the water bath at 37 ^{o}C for 30 s. The characteristics of semen and spermatozoa were evaluated after thawing.

Parameters Observed

Parameters observed were (1) the macroscopic characteristics of fresh semen (volume, color, consistency, and pH) and microscopic characteristics of spermatozoa (motility, membrane integrity, viability, and abnormality), (2) The spermatozoa quality during cryopreserva-

tion process, i.e., motility, viability, and membrane integrity. The pH was measured by using pH-indicator paper 6.4-8.0 (Merck).

Data Analysis

The data on the characteristics of fresh semen and spermatozoa were analyzed by average test, while the data on the quality of spermatozoa during cryopreservation were analyzed by analysis of variance. If there was a difference between groups then the analysis was continued with Duncan's Multiple Range Test (DMRT) according to Steel & Torrie (1993). The data were processed by using SPSS version 19.

RESULTS AND DISCUSSION

Characteristics of Fresh Spermatozoa of Gembrong Goats

The observation of fresh semen and spermatozoa characteristics of Gembrong goat was conducted to test whether the semen is feasible to be processed for cryopreservation or freezing and to determine the level of dilution that will be used. The data about the characteristics of fresh semen of Gembrong goat collected by using artificial vagina showed that the semen had a good quality and met the requirement for cryopreservation (Table 1). The criteria of fresh semen that can be used for cryopreservation and as a base for determination of male fertility with the very good category must have the percentage of sperm motility >50% (Pezzanite *et al*., 2012), sperm concentration of 2 x 10^9/mL, minimum percentage of sperm viability of 80%, and the percentage of sperm abnormality is not more than 15% (Tambing *et al*., 2000).

The volume of semen of Gembrong goat found in this experiment showed a value similar to the previously reported in Ettawah crossed Goat i.e., 0.68±0.18 mL (Rizal, 2008). However, the volume of semen found in Gembrong goat in this experiment was lower as compared those reported in Nubian goat (1.50±0.50 mL) and Nubian crossed Goat (Nubian x PE) (1.33±0.29 mL) (Husin *et al.,* 2007), in the Boer goat (0.83±0.29 mL by Husin *et al.*, 2007 and 0.8±0.2 mL by Kostaman & Sutama, 2006), and in Saanen goat (1.13±0.37 mL) (Tambing *et al.*, 2003). The volume of semen found in this experiment was in the normal range i.e., 0.1-1.5 mL (Jainudeen *et al.*, 2000). Furthermore, Suharyati and Hartono (2013) stated that the volume of semen of Boer goat ranged 0.77-1.13 mL. According to Ax *et al.* (2000), the volume of semen is different according to breed, age, body size, season, feeding level, and the frequency of semen collection.

Table 1. Characteristics of fresh semen of Gembrong goat

Parameter	
Volume (mL)	0.5±0.1
Color	Creamy and milky
Consistency	medium-viscous
pH	6.4±0.0
Sperm concentration (.....x 10^6)	6,731±785
Sperm abnormality (%)	5.74±1.59

The results of this experiment showed that the fresh semen of Gembrong goat had a creamy and milky color with a medium to viscous consistency with the sperm concentration of 6,731±785 x10^6/mL. Some previous studies reported that the color of fresh semen of Ettawah crossed goat was milky white with a consistency a little bit viscous, and sperm concentration of 4,148.57±198.60 x10^6/mL (Rizal *et al*., 2008). However, Isnaini (2011) reported that the color of fresh semen of Boer goat was turbid white with a consistency of a little bit viscous, and sperm concentration of 3.387±230.32 x10^6/mL. According to Ax *et al.* (2000), the color of fresh semen in goat was milky white to creamy, but in Boer goat, the color of semen was white to creamy (Kostaman and Sutama, 2006).

The average pH of fresh semen of Gembrong goat was 6.4. Some previous reports stated that the pH of the semen in Ettawah crossed Goat was 6.8 (Yani *et al*., 2001), Saanen goat was 7.13±0.24 (Tambing *et al*., 2003), Nubian goat was 6.67±0.76, Nubian Crossed Goat (Nubian x PE) was 6.50±0.50, and Boer goat was 6.83±0.29 (Husin *et al*., 2007). The range of semen pH found in this experiment was in the normal range of 5.9-7.3 that was reported in goat (Garner and Hafez, 2000).

The average of abnormal spermatozoa found in this experiment was 5.74±1.59%. The result found in this experiment was higher than that reported by Suharyati and Hartono (2013), where the percentage of abnormal spermatozoa in Boer goat was 1.11%-2.34%. However, the result found in this experiment was lower than those reported by some previous studies, where the percentage of abnormal spermatozoa in Ettawah crossed Goat was 7.12±0.93% (Rizal *et al*., 2008), Kacang goat was 8.6±2.4% (Bintara, 2011), Rayini goat was 9.85±0.25% (Zamiri & Heidari, 2006), Majorera goat was 6.5±1.2% (Batista *et al*., 2009), and Nubian as well as Nubian crossed goats (Nubian x PE) were 12.83±0.81 and 12.34±2.77%, respectively (Husin *et al*., 2007). Senger (2005) reported that each fraction of semen, both obtained from cauda epididymis and ejaculate, contained abnormal spermatozoa around 5%-15% and the decreased in fertilization capability was occurred when the abnormal sperm morphology was more than 20%. The percentage of abnormal spermatozoa found in this experiment was still in the range of normal value.

The Characteristics of Spermatozoa during Cryopreservation Process in Gembrong Goat

The process of cryopreservation strongly affected the characteristics of spermatozoa in Gembrong goat (Figure 1). The results of the experiment showed that the percentages of sperm motility, viability, and membrane integrity of Gembrong goat during cryopreservation process were decrease. After thawing, the percentage of spermatozoa motility decreased significantly ($P<0.05$) as compared to those observed in the early stage and at equilibration stage. The percentages of sperm viability and membrane integrity of Gembrong goat during cryopreservation showed a similar pattern to the percentage of progressive motility of spermatozoa.

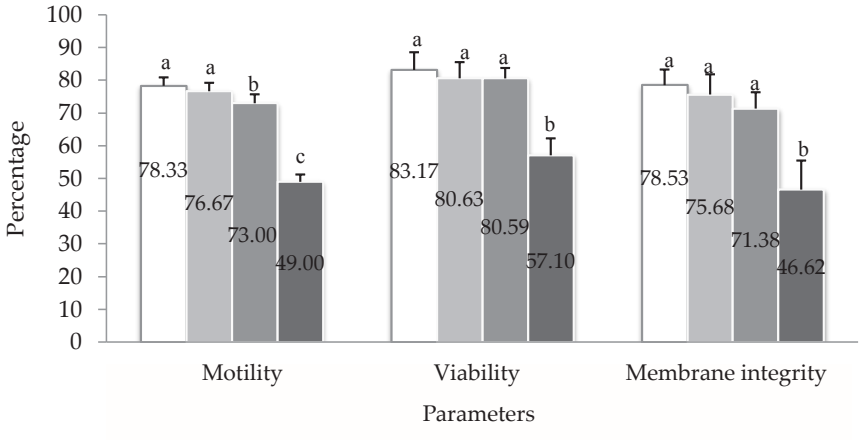

Figure 1. Percentage of sperm motility, viability, and sperm membrane integrity during semen cryopreservation in Gembrong goat. In each stage of cryopreservation, bar with different superscripts (a,b,c) indicated a significant difference (P<0.05).
□ Fresh ■ Before Equilibration ■ After Equilibration ■ Post Thawing

This result showed that the spermatozoa of Gembrong goat were sensitive to cold shock due to the decreased temperature during cryopreservation process. Spermatozoa experienced cellular, biochemical, and osmotic changes during maturation process in epididymis and after the addition of semen plasma at the time of ejaculation. These changes and a dramatic decrease in membrane lipid composition would affect the biological characteristics of spermatozoa at the time of cryopreservation, the permeability of cryoprotectant, and the changes in plasma membrane phase during cooling and freezing (Yeung *et al*., 2006).

Cryopreservation could cause the destruction of membrane functions including the increase in membrane fluidity that is worsen by osmotic shock in the membrane that is occurred when the cell experienced an extreme dehydration during the process of cooling and freezing (Shadan *et al*., 2004). The cryopreservation process causes the decrease in the integrity of the acrosome, the destruction of spermatozoa function and changes in the membrane fluidity, phospholipid and protein aggregation, that are associated with the decrease in enzyme activity, motility, viability, and the ability as well as the capacity to fertilize the ovum (Januskauskas *et al*., 2003). Hermansson and Axner (2007) reported the decrease in motility and acrosome integrity after thawing. The cooling process in cryopreservation of spermatozoa could suppress metabolic activity of the spermatozoa cells that could decrease energy consumption significantly and cold shock sensitivity that are indicated by the irreversible loss of permeability and integrity of plasma membrane of the spermatozoa that could cause the problem and finally the mortality of the spermatozoa (Guthrie *et al*., 2011).

The decreases in the percentage of sperm motility and viability, and integrity of sperm membrane during the process of cryopreservation were related to the intracellular changes due to the excretion of water caused by the formation of ice crystal in addition to cold shock. The formation of ice crystals during cryopreservation process of the semen caused the accumulation of electrolytes in the cell that finally caused the destruction of the cell mechanically that eventually affected the metabolism of the spermatozoa. The accumulated electrolyte in the sperm cell would destruct the membrane of spermatozoa so that at the time of thawing, the permeability of sperm membrane would decrease and the spermatozoa would die. The formation of ice crystal could probably be related to the changes in the osmotic pressure in the fraction that did not experience freezing (Watson, 2000).

In addition, at the time of collection and processing of the semen before packing in straw, the spermatozoa have contact with atmospheric air that contains oxygen. This atmospheric contact could cause the increase in oxidative metabolic activity that also causes the increase in free radicals concentration as a product of metabolic process. Free radicals are dangerous to the survival of spermatozoa since free radicals have characteristics that are very reactive to obtain electron by lipid peroxidation reaction. The free radicals will bind and take electron from unsaturated fatty acids in the phospholipids of the cell membrane that could cause chain reaction of lipid peroxidation that can occur continuously (autocatalytic) that finally destruct the whole plasma membrane of the spermatozoa (Holt, 2000).

The percentage of sperm motility after thawing in Gembrong goat was 49.00% with the viability and membrane integrity of 57.10% and 46.62%, respectively. The similar result was also reported by Tambing *et al.* (2003) that average percentage of spermatozoa motility of Saanen goat was 48.33%, and also the percentage of viability and membrane integrity were 58.31% and 49.05%, respectively. Atessahin *et al.* (2008) reported the percentage of motility and membrane integrity of spermatozoa in Angora goat after thawing were 51.88% and 34.43%, respectively. Naijian *et al.* (2013) reported the percentages of motility, viability, and membrane integrity of spermatozoa of Mahabadi goat after thawing were lower, i.e., 46.20%, 51.60%, and 54.59%, respectively. Some previous reports found that the percentage of spermatozoa motility of Florida goat after thawing was 39.94% (Dorado *et al*., 2007), Blanca-Celtiberica goat

was 43.4% (Rabadan *et al*., 2012), and San Clemente goat was 40.04% (Roof *et al*., 2012).

The results of the experiment showed that after freezing and thawing, the spermatozoa of Gembrong goat was in the best quality condition and meet the requirement for usage in artificial insemination or embryo production in vitro. This good result was related to the high percentage of sperm motility (i.e., 49.00±2.24%). Frozen semen that meets the requirement for artificial insemination must have the percentage of sperm motility at least 40% (Hafez & Hafez, 2000).

To obtain a better semen quality in the process of cryopreservation of semen of Gembrong goats, it is recommended to use some methods of cryopreservation with different compositions of extender.

CONCLUSION

Semen and spermatozoa of Gembrong goats have good quality and meet the requirement for cryopreservation. The characteristics of Gembrong semen are also in the good conditions at the stage of after freezing and after thawing, and meets the requirement for use in artificial insemination or embryo production *in vitro*.

ACKNOWLEDGEMENT

This study was funded by Indonesian Center for Agricultural Biotechnology and Genetic Resources (DIPA no. 018.09.2.237221/2013 dated December 5th 2012). Authors would like to thank Imaniyanto from Reproduction Laboratory in Goats Research Station for her technical assistance.

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